Electronically Filed

DECLARATION UNDER 37 C.F.R. §1.132 Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450		
	Attorney Docket No.	STAN-297
	Confirmation No.	1285
	First Named Inventor	OMARY, M. BISHR
	Application Number	10/552,949
	Filing Date	July 27, 2006
	Group Art Unit	1634
	Examiner Name	Myers, Carla J
	Title: "KERATIN 8 AND 18 MUTATIONS ARE RISK FACTORS FOR DEVELOPING LIVER DISEASE OF MULTIPLE ETIOLOGIES"	

The Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Dr. Bishr Omary, do hereby declare as follows:

I am a co-inventor of the above captioned patent application. The following data was developed in my laboratory and with my collaborators, and supports the claims of the present application. We found that *KRT8/KRT18* are important susceptibility genes for ALF development. The presence of K8/K18 variants predisposes to an adverse acute liver failure (ALF) outcome.

Keratins (K), the largest subgroup of the intermediate filament cytoskeleton, are found predominantly in epithelial cells and skin appendages. They are subdivided into type-I (K9-28, K31-40) and type-II (K1-8, K71-86) proteins; and members of both types co-polymerize to produce filamentous cytoplasmic arrays. Keratins are tissue-specific proteins and their mutations result in a variety of organ-specific human diseases. Adult hepatocytes are unique in that they express K8/K18 only, whereas other digestive-organ epithelial cells exhibit a more complex keratin-expression pattern.

Acute liver failure (ALF) involves ~2000 US cases/year and is characterized by sudden loss of hepatocyte function in previously liver-healthy subjects that may lead to profound morbidity and mortality. Drug-induced liver toxicity is the most common cause of adult ALF mainly because of acetaminophen (APAP) poisoning (~1/2 of US ALF cases). Other frequent ALF etiologies include hepatitis B (7%), indeterminate (14%), autoimmune (5%) and drugs other than APAP (11%). Liver transplantation represents the only effective ALF therapy. Although the pathogenesis of ALF is poorly understood, human genetic variations are predictably important prognosticators as demonstrated for chronic liver diseases. The experiments described herein

demonstrate that K8/K18 mutations predispose to acute liver injury, and have prognostic implications once ALF develops.

METHODS

Human Subjects: We utilized clinical data and genomic DNA from 346 adult ALF patients prospectively identified and consecutively enrolled (2003-2007) at 21 tertiary US centers participating in the Acute Liver Failure Study Group (ALFSG). Patients were selected based on standard ALF criteria that include presence of coagulopathy (INR≥1.5) and hepatic encephalopathy within 26 weeks of the first symptoms in previously liver-healthy individuals. All subjects provided informed consent and the study was approved by the Human Subjects Committees of all participating centers. To estimate the frequency of selected K8 variants in African-Americans, 236 non-ALF subjects were analyzed (136 healthy blood-bank donors and 100 Coriell Institute for Medical Research donors). Previously-published cohorts of healthy Caucasian subjects were used as controls. Nearly 10% of the enrolled ALF patients received N-acetylcysteine as part of an independent drug-efficacy study. However, there was no association between N-acetylcysteine administration and any of our findings.

ALF etiology, patient management and liver transplant candidacy were determined at the enrollment sites and all diagnoses were reviewed at the ALFSG central site. Indeterminate ALF etiology was assigned when extensive clinical/radiographic/laboratory-workup revealed no known ALF cause. Disease outcomes were evaluated 3-weeks after study admission. Race/ethnicity were determined using standard criteria. For outcome analysis, hepatic encephalopathy/coma was scored with a standard scale of 1-4. For bilirubin, INR and creatinine, the maximum value from the pre-admission and admission data was used. When patients died during the observation period, the maximal coma score was set as 4. Of 346 initially-enrolled patients, two were excluded (one did not meet entry criteria and one because of inability to amplify the DNA).

Genetic Analysis: Genomic DNA was isolated from EDTA-anticoagulated blood with a DNeasy-tissue-kit (Qiagen). All 15 total K8/K18 exons and the exon-intron boundaries were PCR-amplified using established primers. Amplified samples were screened for the presence of heterozygous variants with a WAVE DNA Fragment Analysis system (Transgenomics). Samples yielding a shifted elution pattern were purified then sequenced bi-directionally. Assignment of K8/K18 variants was based on the mRNA sequences NM 002273.2/NM 000224.2.

Animal Experiments: Transgenic mice expressing human K8 R341H were generated as described for the mice expressing human K8 G62C that we generated previously. A wild-type (WT) 12-kb genomic clone was used to generate the K8 R341H transgene. A BamHI-SalI

fragment was subcloned into pcDNA3.1. The Arg-to-His mutation (CGT→CAT) was introduced using a Quickchange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The mutated K8 fragment was complemented with a K8 segment excised from the original clone.

Mice (all FVB/n strain) expressing similar levels of human K8 WT, G62C or R341H were studied. After overnight fasting, mice (2month-old males) were injected intraperitoneally with APAP (600 mg/kg mouse weight). Animals were sacrificed after 18h followed by blood collection to measure alanine/aspartate aminotransferases (ALT/AST). Dissected livers were fixed for hematoxylin&eosin (H&E) staining. H&E-stained liver sections were scored as: 1, no obvious pathology; 2, isolated hepatocyte swelling/inflammation/necrosis; 3, distinct necrotic areas/inflammation/swelling; 4, confluent necrotic areas or isolated hemorrhagic areas; 5, confluent necrotic areas or prominent hemorrhagic areas; 6, confluent hemorrhagic areas. The Mann-U-Whitney test and two-tailed student's t-test were used to assess significance of the liver enzymes and histology scores. Animal experiments were approved by the Institutional Animal Care Committee.

Statistical Methods: We analyzed the association of K8/K18 variants with ALF development and outcome. The frequency of individual K8 variants and total significant K8/K18 variants was first compared between ALF patients and controls as well as ethnic groups using Chi-square test. Association of K8/K18 variants with race/ALF etiology/enrolment site was examined using two-tailed Fisher's exact test. Univariate logistic regression analysis was performed to assess the association of K8/K18 variants with different variables and outcomes (STATA software, version-10; stata.com). Since the presence of selected K8/K18 variants affected spontaneous survival (without liver transplantation), a multivariate analysis was performed to adjust for the simultaneous effects of other variables. We used a multivariate-logistic-regression model with age; African-American race (yes/no); and ALF etiology [acetaminophen-related (yes/no), ischemic-related (yes/no) and "other category" of ALF] as predictors. We determined estimates and 95%-confidence intervals for the adjusted odds ratios. All p-values in the logistic regression analysis were computed with a standard Wald test.

Cell Culture and Biochemical Methods: The K8 G434S/S432A cDNAs were previously described. The antibodies (Ab) that were used were directed to: wild-type K8/K18; K8 pS432 (Ab-2D6 and 5B3); human K18 pS53; K18 D237 (K18 apoptotic fragment); K8 R341H. BHK-21 cells were transiently co-transfected with K8 WT/G434S/S432A together with K18 WT. Cells were treated +/-okadaic acid (1h, 0.5µg/ml) and harvested two days after transfection. Total cell/liver tissue lysates were prepared using Laemmli sample buffer. The insoluble keratin-enriched fraction was isolated by high-salt-extraction. Equal amounts of proteins were

separated by SDS-PAGE followed by immunoblotting.

RESULTS

K8/K18 Variants are Overrepresented in ALF and in Subjects with Unique Ethnic Backgrounds: The composition of the ALF cohort (age, male/female ratio, frequency of ALF etiologies) is comparable with previously published data, while spontaneous survival was slightly higher in our cohort (57 vs. 45%, p<0.001). Caucasian and African-American patients were the most common races, making up 73% and 12% of the cohort, respectively. Patients with APAP and ischemic ALF are more likely to survive without transplantation versus other ALF etiologies (p<0.01 for each of the subgroups).

Mutational analysis of the entire coding regions of the *KRT8/KRT18* genes in 344 ALF patients identified 15 amino-acid-altering and 11 non-amino-acid-altering K8/K18 heterozygous variants (Table 1 and Table 2).

Table 1

Variant	Nucleotide	# of patients (%)
K8 Y54H	TAT→CAT	1 (0.3)
K8 G62C	GGC→TGC	4 (1.2)
K8 T153A	**ACT→GCT**	**1 (0.3)***
K8 R341H	CGT→CAT	23 (6.7)**
K8 V380I	GTC→ATC	2 (0.6)
K8 G422V	**GGT→GTT**	**1 (0.3)**
K8 G434S	GGC→AGC	11 (3.2)
K8 I466V	**ATC→GTC**	**1 (0.3)***
K18 V48A	GTG→GCG	**1 (0.3)**
K18 G69A	GGG→GCG	**1 (0.3)**
K18 Δ65-72	N/A	1 (0.3)
Total		**45 (13.1)**

All described variants are heterozygous. Keratin variants were assigned as significant based on previous human association studies or documented biological effects. Five patients harbored two independent amino-acid altering K8 variants (2 K8 A319S+R341H; 1 K8 T153A+R341H; K8 R341H+I466V; 1 K8 I63V+V380I).
* Represents compound heterozygous variants.
** Two of the highlighted variants represent compound heterozygous amino-acid-altering variants.

Bold lettering signifies novel variants which were not previously described.

There were 10 newly identified variants, five of which led to amino acid substitutions in residues that are conserved across mammalian species except for K8 T153. Eleven K8/K18 variants involving 45 patients were assigned as biologically-relevant based on previous human association studies or documented biological effects.

Of the 11 K8/K18 variants, K8 R341H was the most common (seen in 23 subjects), and as shown previously was uniformly associated with the intronic K8 IVS7+10delC. Combining findings herein with prior published data, patients with K8 R341H are more likely to also carry K8 A319S polymorphism (p<0.0001). Interestingly, autoimmune ALF patients had the highest frequency of K8/K18 variants while no K8/K18 variants were seen in subjects with rare ALF

etiologies. When the analyzed races were considered separately, African-American ALF patients had the highest frequency of K8/K18 variants (e.g., 31.7% versus 9.1% in Caucasians, p=0.0003) with K8 G434S then A333A being the most common (Table 2).

Table 2

	Subgroup	Y54H	A333A	R341H	G434S	Total*
ALF	Caucasian	0/252	0/252[7]	18/252[1]	0/252[8]	23/252[2,9]
	African-American	1/41	5/41[7]	0/41	10/41[2,8]	13/41[9]
	APAP	0/167	0/167	12/167	3/167	21/167
	Total	1/344	6/344	23/344	11/344	45/344
Control¶	Caucasian	0/338[4]	0/338[5]	23/727[1]	0/338[6]	9/268[3]
	African-American	10/245[4]	31/245[5]	4/245	25/245[2,6]	N.A.

*Includes all variants which are likely biologically significant based on previous human association studies or their biological effects.

¶The "control" group consists of a cohort of 236 African-Americans analyzed in this study (see Methods section for details) as well as previously published cohorts of liver healthy African-American or Caucasian subjects.[8,18,19]

[1]p<0.0001 (7.1% vs. 3.2%; OR 2.35, 95%CI 1.2-4.6)
[2]p<0.02 (24.4% vs. 10.2%; OR 2.84, 95%CI 1.1-6.8)
[3]p<0.0001 (9.1% vs. 3.4%; OR 2.89, 95%CI 1.3-7.2)
[4]p=0.0002 (0% vs. 4.1%)
[5]p<0.0001 (0% vs. 12.7%)
[6]p<0.0001 (0% vs. 10.2%)
[7]p<0.0001 (0% vs. 12.2%)
[8]p<0.0001 (0% vs. 24.4%)
[9]p=0.0003 (9.1% vs. 31.7%; OR 4.62, 95%CI 1.9-10.7)

To determine the frequency of K8 variants in African-Americans overall, we analyzed K8 exons 1,6 and 8, which include the K8 mutation hot-spots, in 236 control African-American subjects. We identified 7 amino-acid-altering K8 variants (3 novel), and 7 non-amino-acid-altering variants (5 novel). K8 A333A and K8 G434S were the most frequent variants (>10%), while other K8 variants (Y54H/E576E/V480I) appear to be unique to African-Americans.

Next, we compared the frequency of K8/K18 variants in ALF patients versus controls (Table 2). In Caucasians, the frequencies of total K8/K18 variants and the K8 R341H variant were significantly higher than in controls (9.1% versus 3.4%, p<0.0001 for total variants; and 7.1% versus 3.2%, p<0.0001 for K8 R341H) (Table 2). Similarly, K8 G434S was more frequent in African-American ALF patients versus African-American controls (p<0.02).

Keratin Variants Associate with Adverse ALF Outcome: We used univariate analysis to examine the association of K8/K18 variants with clinical prognostic markers related to ALF patients. K8 A333A/A338A, K8 G434S and overall K8/K18 variants were unevenly distributed among races. K8 A333A/A338A concentrated in subjects with viral and indeterminate ALF, and patients harboring K8 A333A/A338A had higher bilirubin levels and were significantly more likely to undergo liver transplantation (Table 3).

Table 3. Univariate Analysis

Factor	Total*	R341H	G434S	Comp.	A333A/ A338A	Test
Race	**0.004**	0.145	**0.000**	n.s.	**0.000**	F.E.
Diagnosis	0.09	n.s.	0.19	n.s.	**0.009**	F.E
Center No	n.s.	n.s.	n.s.	n.s.	n.s.	F.E.
Age	n.s.	n.s.	n.s.	n.s.	n.s	L.R.
Coma total	n.s.	n.s.	n.s.	0.147	n.s.	L.R.
Alive	n.s.	n.s.	n.s.	n.s.	n.s.	L.R.
Transpl	0.150	n.s.	0.079	n.s.	**0.045**	L.R.
Surv_no_tx	**0.034**	0.181	0.172	n.s.	0.148	L.R.
Cr max	n.s.	0.10	n.s.	0.069	n.s.	L.R.
INR max	n.s.	n.s.	0.100	n.s.	n.s.	L.R.
Bili max	n.s.	n.s.	0.083	n.s.	**0.041**	L.R.

* Total significant variants (see Table 1).
Comp., Patients harbouring two independent amino-acid altering heterozygous variants; F.E., Fisher Exact test; L.R., Linear regression analysis

Importantly, subjects with K8/K18 variants were less likely to survive ALF without liver transplantation (p=0.034). We used transplant-free survival as an important outcome because other scoring systems (e.g., Model-for-End-Stage-Liver-Disease; MELD) do not apply for ALF. To address whether the presence of K8/K18 variants affected patient survival without liver transplantation, we performed a multivariate analysis adjusted for age, African-American race and the APAP-related and ischemic ALF etiologies. Presence of K8 R341H associated significantly with lower survival without transplantation, while patients with K8/K18 variants exhibited a trend towards lower survival both in the overall cohort and APAP subgroup (Table 4).

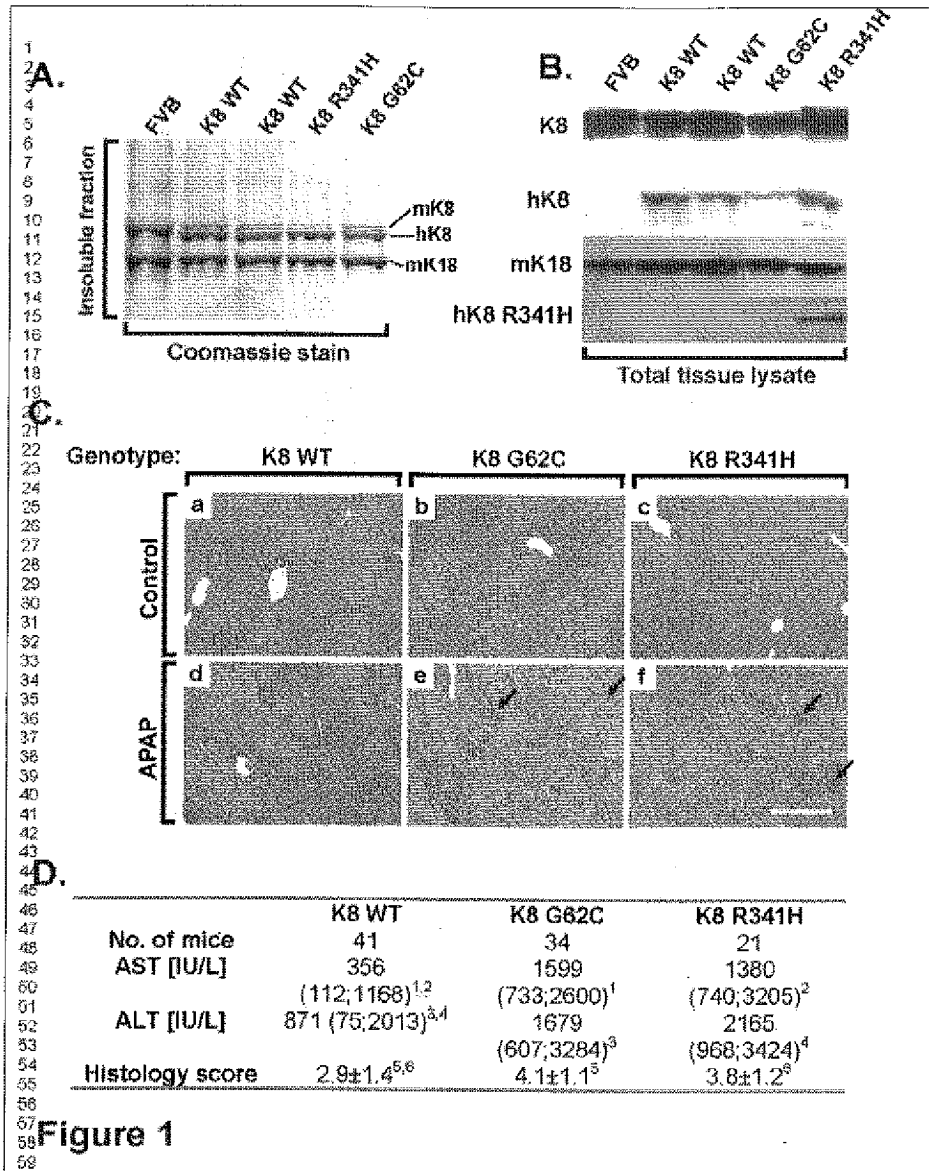
Table 4. Multivariate Analysis of the impact of K8/K18 variants on survival without transplantation

Subgroup	Variants	% Nonsurvivors/Total With Variants	% Nonsurvivors/Total Without Variants	p-value	OR	95% CI
Total	Significant	58	41	<0.1	1.8	0.90-3.78
Total	R341H	57	42	<0.05	2.6	1.01-6.61
Acetaminophen	Significant	43	22	0.05	2.6	0.99-6.77
Acetaminophen	R341H	42	23	0.14	2.5	0.75-8.56
Caucasians	Significant	57	37	0.02	3.1	1.19- 7.98
Caucasians	R341H	61	37	<0.02	3.6	1.23-10.51

Analysis was adjusted for age, African-American race (yes/no) and the acetaminophen-related (yes/no) and ischemic (yes/no) ALF etiology. Nonsurvivors were defined as patients who either died or received a liver transplantation. Of note, we used the initial MELD score to determine if the transplanted group differed from those who died without transplant and found no statistically significant difference between both groups (p=0.09 in all subjects, p=0.40 in Caucasians). In contrast, patients who survived transplant-free displayed significantly lower initial MELD scores than patients who were either transplanted or who died without transplant (27.6 ± 8.0, 34.2 ± 8.0 and 36.4 ± 8.3, respectively; p<0.001 in both cases overall and in Caucasians). Although transplant criteria differ among transplant centers, and MELD scores are not used for listing patients with ALF,[26] these MELD estimations help justify using transplant-free survival as an important endpoint indicative of less severe disease.

In Caucasian patients, there was a significant association between the presence of either K8 R341H or significant K8/K18 variants and lower survival without transplantation (Table 4). Hence, K8/K18 variants appear to predispose to the development of ALF and are also associated with an adverse outcome once ALF develops.

K8 R341H Mice are More Susceptible to APAP-Induced ALF: In order to further substantiate the importance of K8 R341H in human ALF, we generated transgenic mice that express human K8 R341H and tested their susceptibility to APAP-induced liver injury. Expression of the human K8 transgene was confirmed biochemically. As controls, we used mice that express WT human K8, or mice that express human K8 G62C which is also found in some ALF patients, and predisposes to apoptotic liver injury induced by Fas ligand. Human K8 protein expression was comparable in the three genotypes and there was no evidence of liver pathology under basal conditions. However, APAP challenge led to more prominent liver injury (necrosis, hemorrhage, serologic hepatitis) in the K8 G62C and R341H genotypes as compared with K8 WT.



	K8 WT	K8 G62C	K8 R341H
No. of mice	41	34	21
AST [IU/L]	356 (112;1168)[1,2]	1599 (733;2600)[1]	1380 (740;3205)[2]
ALT [IU/L]	871 (75;2013)[3,4]	1679 (607;3284)[3]	2165 (968;3424)[4]
Histology score	2.9±1.4[5,6]	4.1±1.1[5]	3.8±1.2[6]

Figure 1

Figure 1: K8 G62C and K8 R341H predispose to acetaminophen-induced liver injury (A) Insoluble protein fraction was extracted from livers of non-transgenic animals (lane 1) and mice overexpressing WT human K8 (lanes 2,3; two independent livers), K8 R341H (lane 4) or K8 G62C (lane 5) followed by visualization of proteins by Coomassie staining. Note the down-regulated mK8 levels in transgenic animals overexpressing human K8 in order to maintain steady total K8 levels as previously described.[11] (B) Total liver homogenates were prepared from the same mice used in Panel A. The homogenates were blotted with antibodies to the indicated proteins. (C) H&E-stained sections depict the liver histology of mice expressing human K8 WT (a,d), K8 G62C (b,e) or K8 R341H (c,f) prior to (a-c) and after APAP administration (d-f). While all non-treated mice appear histologically normal, APAP-exposed K8 G62C and K8 R341H animals exhibit more pronounced liver necrosis and hemorrhage (arrows in e,f). Scale bar, 200 µm. (D) After APAP administration, K8 G62C and R341H mice develop increased liver serologic and histological scores when compared to mice overexpressing K8 WT. The results are expressed as means ± SD (histology score) or median with first and third quartile (liver enzymes). The matching superscript numbers indicate groups with significant differences in their liver enzyme levels/histological scores. The corresponding p-values are:[1]p=0.005; [2]p=0.007; [3]p=0.02; [4]p=0.01; [5]p=0.002; [6]p=0.04

Functional Analysis of K8 Variants: We addressed potential functional consequences of the K8 G434S variant in cell transfection models. Prior findings showed that G434S limited the ability of K8 to serve as a substrate for the mitogen-activated protein kinase. We hypothesized that this is most likely related to a conformational change that affects phosphorylation of the previously-characterized physiologic K8 S432 phospho-site. This was substantiated using two independent anti-K8-pS432 antibodies whose reactivity is ablated when S432 or G434 is mutated in transfected cells. Furthermore, K8 S432A and G434S lead to increased caspase-mediated degradation of the partner K18 when compared with K8 WT which lends further support to the importance of the K8 G434 residue in the conformational state of K8 and how it interacts with its binding partner K18.

The overall frequency of biologically-relevant K8/K18 variants in our cohort (13.1%) was similar to that in patients with chronic end-stage liver disease (13.4%), but significantly higher than in blood-bank donors (3.7%). This highlights the importance of *KRT8/KRT18* gene variants in both acute and chronic liver disease. K8 R341H is the most frequent amino-acid-altering variant in Caucasians and was found at increased frequency in Caucasian ALF patients versus ethnically-matched controls ($p=0.01$). K8 R341H also associates exclusively with the intronic K8 IVS7+10delC deletion, as confirmed herein, and appears to segregate with K8 R319S. This suggests that nucleotide changes at one site trigger errors at other sites. In Caucasians, K8 R341H/G62C are the two most common variants, and the human association data are strongly supported by transgenic animal models that express these individual mutations.

K8 G434S was the most common variant in African-Americans and associates preferentially with ALF versus ethnically-matched controls ($p<0.02$). Five variants were seen at significantly higher frequencies in African-American versus Caucasian controls (K8 Y54H/A333A/E376E/G434S/V480I) including Y54H/G434S which were identified in earlier studies. Collectively, these variants were observed in 33% of African-Americans and <1% of Caucasians.

The autoimmune and APAP ALF subgroups had unique K8/K18 variant-related associations. Autoimmune ALF patients had the highest percentage of K8/K18 variants while in the ALF-subgroup afflicted with APAP poisoning, presence in Caucasians of any K8/K18 variant or of K8 R341H is significant when compared with Caucasian controls (10.1 versus 3.4%, $p=0.01$; and 7.2 versus 3.2%, $p<0.05$, respectively).

Our findings highlight the importance of genetic variations in ALF. We used a large prospectively-identified cohort of ALF patients to identify *KRT8/KRT18* as important susceptibility genes for ALF development. K8/K18 variants predisposes to an adverse ALF outcome in humans and animals which raises the possibility for the utility of K8/K18 variants as

prognostic biomarkers. Collectively, these findings provide a unique link of the cytoskeleton to ALF.

I hereby declare that all statements made herein of my own knowledge are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date: August 17, 2009

By 

Bishr Omary, M.D.